

APPLICATION UNDER UNITED STATES PATENT LAWS

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Invention: Sequences which code for the sigE gene

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SPECIFICATION

Sequences which code for the sigE Gene

Field of the Invention

The invention provides nucleotide sequences from coryneform bacteria which code for the sigE gene and a process for the fermentative preparation of amino acids using bacteria in which the sigE gene is enhanced.

Prior Art

L-Amino acids are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and especially in animal nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and produce amino acids are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of *Corynebacterium* strains which produce L-amino acid, by

amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

Object of the Invention

The inventors had the object of providing new measures for improved fermentative preparation of amino acids.

Summary of the Invention

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. Lysine is particularly preferred.

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the sigE gene, chosen from the group consisting of

- a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the polynucleotides of a) or b), and
- d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of sigma factor E.

The invention also provides the above-mentioned polynucleotide, this preferably being a DNA which is
5 capable of replication, comprising:

- (i) the nucleotide sequences shown in SEQ ID No. 1, SEQ ID NO. 3 or 4, or
- (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration
10 of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally
- (iv) sense mutations of neutral function in (i).

15 The invention also provides

a polynucleotide, in particular DNA, which is capable of replication and comprises the nucleotide sequence as shown in SEQ ID No. 1;

20 a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the polynucleotide according to the invention, in particular a shuttle vector or plasmid vector, and

25 coryneform bacteria which contain the vector or in which the sigE gene is enhanced.

The invention also provides polynucleotides which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a

- corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

Detailed Description of the Invention

- Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for sigma factor E or to isolate those nucleic acids or polynucleotides or genes which have a high similarity of sequence with that of the sigE gene.

- Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for sigma factor E can be prepared by the polymerase chain reaction (PCR).

- Such oligonucleotides which serve as probes or primers comprise at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable.

"Isolated" means separated out of its natural environment.

- "Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

- The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least 70%, preferably at least 80% and in particular at least 90% to

95% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

5 "Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

10 The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of sigma factor E, and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

15 The invention furthermore relates to a process for the fermentative preparation of amino acids chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine using coryneform bacteria which in particular
20 already produce amino acids and in which the nucleotide sequences which code for the sigE gene are enhanced, in particular over-expressed.

25 The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or allele or of the genes or alleles, using a potent promoter or using a gene or allele which codes for a corresponding enzyme having a high activity,
30 and optionally combining these measures.

The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from

glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium* glutamicum, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

- 10 *Corynebacterium glutamicum* ATCC13032
- Corynebacterium acetoglutamicum* ATCC15806
- Corynebacterium acetoacidophilum* ATCC13870
- Corynebacterium thermoaminogenes* FERM BP-1539
- Corynebacterium melassecola* ATCC17965.
- 15 *Brevibacterium flavum* ATCC14067
- Brevibacterium lactofermentum* ATCC13869 and
- Brevibacterium divaricatum* ATCC14020

and L-amino acid-producing mutants or strains prepared therefrom.

- 20 The new sigE gene from *C. glutamicum* which codes for the enzyme sigma factor E has been isolated.

- To isolate the sigE gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in *Escherichia coli* (*E. coli*). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: *Gene und Klone, Eine Einführung in die Gentechnologie* (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the *E. coli* K-12 strain W3110 set up in λ vectors by Kohara et al.
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(Cell 50, 495 -508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, 5 Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326 (1992)) in turn describe a gene library of *C. glutamicum* 10 ATCC13032 using the cosmid pH79 (Hohn and Collins, Gene 11, 291-298 (1980)).

To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, 15 Gene, 19:259-268). Suitable hosts are, in particular, those *E. coli* strains which are restriction- and recombination-defective. An example of these is the strain DH5 α mc r , which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). 20 The long DNA fragments cloned with the aid of cosmids can in turn be subcloned in the usual vectors suitable for sequencing and then sequenced, as is described e.g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 25 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-30 1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of *C. glutamicum* which codes for the sigE gene and which, as SEQ ID No. 1, is a constituent of

the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the sigE gene product is shown in SEQ ID No. 2.

Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in

the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). The

5 hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i. e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing

10 steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington,

15 UK, 1996).

A 5x SSC buffer at a temperature of approx. 50 - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe.

20 Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim,

25 Mannheim, Germany, 1995) a temperature of approx. 50 - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to the sequence of the probe

30 employed can be isolated by increasing the hybridization temperature stepwise from 50 to 68°C in steps of approx. 1 - 2°C. Further instructions on hybridization are obtainable

on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany,

35 Catalogue No. 1603558).

Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait:

- 5 Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

In the work on the present invention, it has been found that coryneform bacteria produce amino acids in an improved manner after over-expression of the sigE gene.

- 10 To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act
15 in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative amino acid production. The expression is likewise improved by measures to prolong the life of the mRNA. Furthermore, the enzyme activity is also increased by
20 preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be
25 achieved by changing the composition of the media and the culture procedure.

- Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)),
30 Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in EP 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132
35 (1994)), in LaBarre et al. (Journal of Bacteriology 175,

1001-1007 (1993)), in WO 96/15246, in Malumbres et al.
(Gene 134, 15 - 24 (1993)), in JP-A-10-229891, in Jensen
and Hammer (Biotechnology and Bioengineering 58, 191-195
(1998)), in Makrides (Microbiological Reviews 60:512-538
5 (1996)) and in known textbooks of genetics and molecular
biology.

By way of example, for enhancement the sigE gene according
to the invention was over-expressed with the aid of
episomal plasmids. Suitable plasmids are those which are
10 replicated in coryneform bacteria. Numerous known plasmid
vectors, such as e.g. pZ1 (Menkel et al., Applied and
Environmental Microbiology (1989) 64: 549-554), pEKEx1
(Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen
et al., Gene 107:69-74 (1991)) are based on the cryptic
15 plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such
as e.g. those based on pCG4 (US-A 4,489,160), or pNG2
(Serwold-Davis et al., FEMS Microbiology Letters 66, 119-
124 (1990)), or pAG1 (US-A 5,158,891), can be used in the
same manner.

20 Plasmid vectors which are furthermore suitable are also
those with the aid of which the process of gene
amplification by integration into the chromosome can be
used, as has been described, for example, by Reinscheid et
al. (Applied and Environmental Microbiology 60, 126-132
25 (1994)) for duplication or amplification of the hom-thrB
operon. In this method, the complete gene is cloned in a
plasmid vector which can replicate in a host (typically E.
coli), but not in C. glutamicum. Possible vectors are, for
example, pSUP301 (Simon et al., Bio/Technology 1, 784-791
30 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-
73 (1994)), pGEM-T (Promega corporation, Madison, WI, USA),
pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry
269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen,
Groningen, Holland; Bernard et al., Journal of Molecular
35 Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al, 1991,

Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342). The plasmid vector which contains the gene to be amplified is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene in question.

In addition, it may be advantageous for the production of L-amino acids to enhance, in particular over-express one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the sigE gene.

Thus, for example, for the preparation of L-amino acids, in addition to enhancement of the sigE gene, one or more genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),

- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
- 5 • the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512; EP-A-0699759),
- 10 • the lysE gene which codes for lysine export (DE-A-195 48 222),
- the hom gene which codes for homoserine dehydrogenase (EP-A 0131171),
- the ilvA gene which codes for threonine dehydratase (Möckel et al., Journal of Bacteriology (1992) 8065-8072)) or the ilvA(Fbr) allele which codes for a "feed back resistant" threonine dehydratase (Möckel et al., (1994) Molecular Microbiology 13: 833-842),
- 15 • the ilvBN gene which codes for acetohydroxy-acid synthase (EP-B 0356739),
- 20 • the ilvD gene which codes for dihydroxy-acid dehydratase (Sahm and Eggeling (1999) Applied and Environmental Microbiology 65: 1973-1979),
- the zwal gene which codes for the Zwal protein (DE: 25 19959328.0, DSM 13115)

can be enhanced, in particular over-expressed.

It may furthermore be advantageous for the production of L-amino acids, in addition to the enhancement of the sigE

gene, for one or more of the genes chosen from the group consisting of:

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- 5 • the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478; DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE: 1995 1975.7; DSM 13114),
- the zwa2 gene which codes for the Zwa2 protein (DE: 10 19959327.2, DSM 13113)

to be attenuated, in particular for the expression thereof to be reduced.

In addition to over-expression of the sigE gene it may furthermore be advantageous for the production of amino acids to eliminate undesirable side reactions (Nakayama: 15 "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The invention also provides the microorganisms prepared 20 according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of amino acids. A summary of known culture 25 methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/ 30 Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General

- 5 Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil,
10 groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

- 15 Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be
20 used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus.

- 25 The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances.

- 30 Suitable precursors can moreover be added to the culture

medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.

5 Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions,
10 oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within
15 10 hours to 160 hours.

Methods for the determination of L-amino acids are known from the prior art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by ion exchange chromatography
20 with subsequent ninhydrin derivation, or it can be carried out by reversed phase HPLC, for example as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention is used for
25 fermentative preparation of amino acids.

The following microorganism was deposited as a pure culture on 11th April 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany)
30 in accordance with the Budapest Treaty:

- *Corynebacterium glutamicum* DSM5715/pEC-T18mob2sigEexp as DSM 14229.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The isolation of plasmid DNA from *Escherichia coli* and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA). Methods for transformation of *Escherichia coli* are also described in this handbook.

10 The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et al.

Example 1

15 Preparation of a genomic cosmid gene library from *Corynebacterium glutamicum* ATCC 13032

Chromosomal DNA from *Corynebacterium glutamicum* ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product

Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no. 27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 2

20 Isolation and sequencing of the sigE gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Holland, Product Description Zero Background Cloning Kit, Product No. K2500-01), was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

The plasmid preparation of the recombinant clones was carried out with the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig.

The computer-assisted coding region analysis was prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231).

The resulting nucleotide sequence is shown in SEQ ID No. 1.
5 Analysis of the nucleotide sequence showed an open reading frame of 651 base pairs, which was called the sigE gene. The sigE gene codes for a protein of 216 amino acids (SEQ ID NO. 2).

10 The DNA sections lying upstream and downstream of SEQ ID NO. 1, which are shown in SEQ ID NO. 3 and SEQ ID NO. 4, were identified in the same manner. The sigE gene region extended by SEQ ID NO. 3 and SEQ ID NO. 4 is shown in SEQ ID NO. 5.

Example 3

15 Preparation of a shuttle vector pEC-T18mob2sigEexp for enhancement of the sigE gene in *C. glutamicum*

3.1. Cloning of the sigE gene

From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 -1828
20 (1994)). On the basis of the sequence of the sigE gene known for *C. glutamicum* from example 2, the following oligonucleotides were chosen for the polymerase chain reaction (see SEQ ID No. 7 and SEQ ID No. 8).

sigE1:

25 5`TAG TCA CCA CGG TTA AGC CT 3`

sigE2:

5`GCC TTG GTT CTT ACG AAC TG 3`

The primers shown were synthesized by ARK Scientific GmbH Biosystems (Darmstadt, Germany) and the PCR reaction was
30 carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990,

Academic Press) with Taq-Polymerase from Qiagen (Hilden, Germany). With the aid of the polymerase chain reaction, the primers allow amplification of a DNA fragment approx. 2.03 kb in size, which carries the sigE gene.

5 The amplified DNA fragment of approx. 2.03 kb in size which carries the sigE gene was ligated with the TOPO TA Cloning® Kit from Invitrogen Corporation (Carlsbad, CA, USA) in the vector pCR®2.1TOPO (Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)). The E. coli strain
10 Top10 (Grant et al., Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) was then transformed with the ligation batch in accordance with the instructions of the manufacturer of the kit (Invitrogen Corporation, Carlsbad, CA, USA). Selection of plasmid-carrying cells was
15 carried out by plating out the transformation batch on LB Agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 50 mg/l kanamycin. Plasmid DNA was isolated from a
20 transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen (Hilden, Germany) and checked by treatment with the restriction enzyme SphI and EcoRI with subsequent agarose gel electrophoresis (0.8 %). The DNA sequence of the amplified DNA fragment was checked by sequencing. The
25 plasmid was called pCR2.1sigEexp. The strain was called E. coli Top10 / pCR2.1sigEexp.

3.2. Preparation of the E. coli - C. glutamicum shuttle vector pEC-T18mob2

30 The E. coli - C. glutamicum shuttle vector was constructed according to the prior art. The vector contains the replication region reg of the plasmid pGA1 including the replication effector per (US-A- 5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the tetracycline resistance-imparting tetA(Z) gene of the

plasmid pAG1 (US-A- 5,158,891; gene library entry at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with the accession number AF121000), the replication region oriV of the plasmid pMB1 (Sutcliffe, 5 Cold Spring Harbor Symposium on Quantitative Biology 43, 77-90 (1979)), the lacZ α gene fragment including the lac promoter and a multiple cloning site (mcs) (Norranders, J.M. et al. Gene 26, 101-106 (1983)) and the mob region of the plasmid RP4 (Simon et al., (1983) Bio/Technology 1:784-791). 10 The vector constructed was transformed in the E. coli strain DH5 α (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA).

Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., 15 Molecular cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 5 mg/l tetracycline. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by 20 restriction with the restriction enzymes EcoRI and HindIII and subsequent agarose gel electrophoresis (0.8 %). The plasmid was called pEC-T18mob2 and is shown in figure 1.

3.3. Cloning of sigE in the E. coli-C. glutamicum shuttle vector pEC-T18mob2

25 The E. coli - C. glutamicum shuttle vector pEC-T18mob2 described in example 3.2 was used as the vector. DNA of this plasmid was cleaved completely with the restriction enzymes BamHI and SalI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, 30 Mannheim, Germany, Product Description SAP, Product No. 1758250).

The sigE gene was isolated from the plasmid pCR2.1sigEexp described in example 3.1. by complete cleavage with the enzymes BamHI and SalI. The sigE fragment 1930 bp in size

was isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The sigE fragment obtained in this manner was mixed with the prepared vector pEC-T18mob2 and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no. 27-0870-04). The ligation batch was transformed in the E. coli strain DH5 α MCR (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 5 mg/l tetracycline. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and cleaved with the restriction enzymes BamHI and SalI to check the plasmid by subsequent agarose gel electrophoresis. The plasmid obtained was called pEC-T18mob2sigEexp. It is shown in figure 2.

Example 4

Transformation of the strain DSM5715 with the plasmid pEC-T18mob2sigEexp

The strain DSM5715 was transformed with the plasmid pEC-T18mob2sigEexp using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)). Selection of the transformants took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast

extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 5 mg/l tetracycline. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 -927), cleaved with the restriction endonucleases BamHI and SalI, and the plasmid was checked by subsequent agarose gel electrophoresis. The strain obtained was called DSM5715/pEC-T18mob2sigEexp.

Example 5

Preparation of lysine

The *C. glutamicum* strain DSM5715/pEC-T18mob2sigEexp obtained in example 4 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a pre-culture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the pre-culture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2 % (w/v)

The pH was brought to pH 7.4

20

Tetracycline (5 mg/l) was added to this. The pre-culture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this pre-culture

such that the initial OD (660 nm) of the main culture was 0.05. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
$(\text{NH}_4)_2\text{SO}_4$	25 g/l
KH_2PO_4	0.1 g/l
$\text{MgSO}_4 \cdot 7 \text{ H}_2\text{O}$	1.0 g/l
$\text{CaCl}_2 \cdot 2 \text{ H}_2\text{O}$	10 mg/l
$\text{FeSO}_4 \cdot 7 \text{ H}_2\text{O}$	10 mg/l
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
CaCO_3	25 g/l

- 5 The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO_3 autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) was

added. Culturing was carried out at 33°C and 80 % atmospheric humidity.

- After 48 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in Table 1.

10

Table 1

Strain	OD (660 nm)	Lysine HCl g/l
DSM5715/pEC-T18mob2	12.2	13.14
DSM5715/pEC-T18mob2sigEexp	13.07	14.09

Brief Description of the Figures

Figure 1: Map of the plasmid pEC-T18mob2

Figure 2: Map of the plasmid pEC-T18mob2sigEexp

- 15 The abbreviations and designations used have the following meaning.

per: Gene for controlling the number of copies from PGA1

oriV: ColE1-similar origin from pMB1

- 20 rep: Plasmid-coded replication region from C. glutamicum plasmid pGA1

RP4mob: RP4 mobilization site

lacZ-alpha: lacZ gene fragment from E. coli

Tet: Resistance gene for tetracycline

sigE: sigE gene of C. glutamicum

BamHI: Cleavage site of the restriction enzyme BamHI

5 SalI: Cleavage site of the restriction enzyme SalI

sigE: sigE gene of C. glutamicum

BamHI: Cleavage site of the restriction enzyme BamHI

SalI: Cleavage site of the restriction enzyme SalI

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